



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C07K 7 /00	A2	(11) International Publication Number: WO 00/04052 (43) International Publication Date: 27 January 2000 (27.01.00)
(21) International Application Number: PCT/GB99/02292 (22) International Filing Date: 16 July 1999 (16.07.99) (30) Priority Data: 9815505.4 16 July 1998 (16.07.98) GB (71) Applicant (for all designated States except US): ADPROTECH PLC [GB/GB]; Second Floor, Units 7 & 8, The Maltings, Green Drift, Royston, Herts. SG8 5DY (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SMITH, Richard, Anthony, Godwin [GB/GB]; Norfolk House, Haverhill Road, Horseheath, Cambridge CB1 6QR (GB). BRIGHT, Jeremy, Richard [GB/GB]; The Haven, Chesham Road, Berkamstead, Herts. HP4 3AA (GB). STEWARD, Michael [GB/GB]; Whitebeam Cottage, Thomas Walk, Littlebury Green, Saffron Walden, Essex CB11 4XE (GB). COX, Vivienne, Frances [GB/GB]; Bec Cottage, Pounsley Road, Dunton Green, Sevenoaks, Kent TN13 2XP (GB). (74) Agent: DAVIES, Jonathan, Mark; Reddie & Grose, 16 Theobalds Road, London WC1X 8PL (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: POLYPEPTIDE DERIVATIVES		
(57) Abstract <p>It has been found that derivatives of angiogenesis inhibiting proteins may be prepared in which a negative feedback process can be enhanced for therapeutic purposes and which can be targeted to cell membranes and sites of active angiogenesis particularly those of the vascular endothelium. The invention provides a soluble derivative of a polypeptide capable of inhibiting angiogenesis, said derivative comprising a combination of heterologous membrane binding elements covalently associated with the polypeptide so that the derivative acquires affinity for the surface of the vascular endothelium particularly that of growing blood vessels. The soluble polypeptide may be selected from the non-catalytic regions of human plasminogen (within the N-terminal 560 residues of that protein); fragments thereof, particularly those generated by metalloprotease digestion of plasminogen; fragments of related proteins containing kringle domains such as hepatocyte growth factor or apolipoprotein (a), prothrombin, tissue-type plasminogen activator, urinary-type plasminogen activator and hybrids thereof with plasminogen sequences; mutants of the above kringle domains, those containing positively charged to neutral or negatively charged mutations at positions 20, 21, 78 and 79; fragments of collagen, particularly collagen XVIII; fragments of prolactin, the 16kDa N-terminal region of prolactin; neutralising antibodies against receptors for angiogenic mediators; antagonists of integrins involved in angiogenesis; and hybrids, derivatives or muteins thereof. Each membrane binding element with low membrane affinity may have a dissociation constant of 1μM-1mM, and the derivative may incorporate sufficient elements with low affinities for membrane components to result in a 0.01 - 1OnM dissociation constant affinity for specific membranes.</p>		

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

POLYPEPTIDE DERIVATIVES

This invention relates to anti-angiogenic polypeptide derivatives, their use in therapy and methods and intermediates for their production.

Recent studies have identified various polypeptides capable of preventing the growth of tumours through inhibition of the neovascularisation process that provides blood vessels and hence nutrients to the tumour body (angiogenesis). These studies were prompted by earlier observations that some experimental primary tumours could control the growth of either metastatic cell masses derived from them or of secondary tumour inoculates (e.g. E.Gorelik et al, Int. J.Cancer, 27, 617-625, 1978). Furthermore, removal of primary tumours was found to accelerate the growth of metastases (e.g. B.Fisher et al, Cancer Res. 49, 1996-2001, 1989) and conversely, the presence of metastases apparently inhibited the growth of primary tumour (J.M.Yuhas & N.H.Pazmino, Cancer Res. 34, 2005-2010, 1974). These observations prompted the hypothesis that at least two soluble mediators, one stimulatory and the other inhibitory of angiogenesis were being generated by primary tumours and their metastases and were responsible for the apparent communication between the two types of tumour body. O'Reilly et al (Cell, 79, 315-328, 1994) identified one of the soluble inhibitory agents - termed 'angiostatin' - as a fragment of the abundant plasma protein plasminogen. The fragment was isolated from urine and blood of tumour-bearing mice and shown to be derived from the N-terminal region of plasminogen and to contain the first two 'kringle' domains. Subsequent studies showed that the first three kringle domains isolated by limited elastase digestion of plasminogen (L.Sottrup-Jensen et al, Prog.Chem.Fibrinolysis, 3, 191-209, 1978) also had angiostatic activity, that kringle 4 largely lacked it but that kringle 5 alone was a potent inhibitor *in vitro*. (Y.Cao et al, J.Biol.Chem. 271, 29461-29467, 1996, Y.Cao et al, J.Biol.Chem. 272, 22924-22928, 1997). It was further shown that these angiostatic fragments of plasminogen could be generated by cleavage of native plasma plasminogen by the matrix metallo proteases MMP-7 (matrilysin), gelatinase B or MMP-9 (B.C.Patterson & Q.A.Sang, J.Bio.Chem. 272, 28823-28825, 1997) and stromelysin (MMP-3, H.R.Lijnen et al, Biochemistry, 37, 4699-4702, 1998). A requirement for reduction of a protein disulphide bond was also identified (P.Stathakis et al, J.Biol.Chem, 272, 20641-20645, 1997, S.Gately et al, Proc.Natl.Acad.Sci.USA. 94, 10868-10872, 1997).

angiogenic factors at their receptors or by some novel but probably receptor-mediated process. In either case, the mechanisms are such that drug-induced resistance does not seem to occur. It has now been found that derivatives of angiogenesis inhibiting proteins may be prepared in which this negative feedback process can be enhanced for therapeutic purposes and which can be targeted to cell membranes and sites of active angiogenesis particularly those of the vascular endothelium.

WO98/02454 discloses general methods for the preparation of soluble therapeutic polypeptide derivatives with affinity for outer cell membranes through incorporation of a combination of heterologous membrane binding elements with low individual membrane affinities.

The present invention therefore provides a soluble derivative of a polypeptide capable of inhibiting angiogenesis, said derivative comprising a combination of heterologous membrane binding elements covalently associated with the polypeptide so that the derivative acquires affinity for the surface of the vascular endothelium particularly that of growing blood vessels.

By 'heterologous' is meant that the elements are not found in the native full length protein from which the anti-angiogenic protein may be derived.

By 'membrane binding element with low membrane affinity' is meant that the element has moderate affinity for membranes, that is a dissociation constant greater than 0.1 μM , preferably 1 μM -1 mM. The elements preferably have a size <5 kDa.

The derivative incorporates sufficient elements with low affinities for membrane

components to result in a derivative with a high (preferably 0.001 - 10nM dissociation constant) affinity for cell membranes of blood vessel endothelium.

The elements are chosen so as to retain useful solubility in pharmaceutical formulation media, preferably >100µg/ml.

The invention thus promotes localisation of angiostatic agents at cellular membranes and thereby provides one or more of several biologically significant effects with potential advantages in tumour therapy including:

Potency and dosing: In animal studies (e.g T.Boehm et al, *loc cit.*) angiogenic polypeptides have been used at doses which, if extrapolated to man, raise issues of cost, method of delivery and patient compliance in the chronic use of such agents to treat human cancer. If localised at their site of action and on the same surface as their presumed receptors, such agents would have an increased effective concentration and local residence time which reduce both the magnitude and frequency of dosing.

Specificity: The use of membrane targeting agents with selectivity for(e.g) integrins implicated in angiogenesis would reduce the risk of interrupting a normal process elsewhere in the vasculature.

Examples of anti-angiogenic agents which may be modified according to the invention include but are not restricted to the following:

- The non-catalytic regions of human plasminogen (i.e. Within the N-terminal 560 residues of that protein)
- Fragments thereof, particularly those generated by metalloprotease digestion of plasminogen
- Fragments of related proteins containing kringle domains such as hepatocyte growth factor or apolipoprotein (a), prothrombin, tissue-type plasminogen activator, urinary-type plasminogen activator and hybrids thereof with plasminogen sequences
- Mutants of the above kringle domains, for example those containing positively charged to neutral or negatively charged mutations at positions 20, 21 ,78 and 79 (numbering as used by Cao et al, 1997)
- Fragments of collagen, particularly Collagen XVIII
- Fragments of prolactin such as the 16kDa N-terminal region
- Neutralising antibodies against receptors for angiogenic mediators

- Antagonists of integrins involved in angiogenesis

Preferred derivatives of this invention have the following structure:



in which:

A is the soluble anti-angiogenic agent,

each L is independently an optional flexible linker group,

each W is independently a peptidic membrane binding element,

n is an integer of 1 or more and

X is a peptidic or non-peptidic membrane-binding entity which may be covalently linked to any W.

Flexible linker groups are generally short (3 to 10 aminoacids) peptide sequences dominated by non-bulky and relatively hydrophilic residues such as serine, glycine and alanine which may exhibit a beta-turn propensity (and contain Proline) but do not have rigid conformations. Non-rigid linkers other than alpha aminoacids may also be used such as beta-alanine, 6-aminohexanoic acid and alpha-amino, omega-carboxy derivatives of oxyethylene oligomers and polymers.

Peptidic membrane binding elements are preferably 8 to 20 amino acids long and elements W are preferably located sequentially either at the N or C terminus of the soluble polypeptide. The amino acid sequences are linked to one another and to the soluble peptide by linker groups which are preferably selected from hydrophilic and/or flexible aminoacid sequences of 4 to 20 aminoacids; linear hydrophilic synthetic polymers; and chemical bridging groups.

Peptide linkages may be made chemically or biosynthetically by expression of appropriate coding DNA sequences. Non peptide linkages may be made chemically or enzymatically by post-translational modification.

Suitable examples of amino acid sequences comprising peptidic membrane binding elements are disclosed in WO98/02454 and are incorporated herein by reference. They include:

AspGlyProLysLysLysLysLysLysSerProSerLysSerSerGly [SEQ ID No. 10]

GlySerSerLysSerProSerLysLysLysLysLysLysProGlyAsp [SEQ ID No. 11]

SerProSerAsnGluThrProLysLysLysLysLysArgPheSerPheLysLysSerGly [SEQ ID No. 12]

AspGlyProLysLysLysLysLysLysSerProSerLysSerSerLys [SEQ ID No. 13]

SerLysAspGlyLysLysLysLysLysLysSerLysThrLys [SEQ ID No. 14]

These are examples of electrostatic switch sequences and they may be combined with amino acid sequences derived from ligands of integral membrane proteins known to be associated with vascular endothelium and angiogenic processes. For example, the $\alpha v \beta 3$ integrins are receptors for vitronectin and have ligands containing the RGD sequence.

Such peptides have been identified by phage display techniques (e.g E.Koivunen et al, Biotechnology, 13, 265-270, 1995) and include the disulphide-constrained sequence AlaCysAspCysArgGlyAspCysPheCysGly [SEQ ID No. 15].

Another example is AspGlyProSerGluIleLeuArgGlyAspPheSerSer [SEQ ID No. 16] derived from human fibrinogen alpha chain, which binds to the GpIIb/IIIa membrane protein in platelets. In general, binding sequences from random chemical libraries such as those generated in a phage display format and selected by biopanning operations *in vitro* (G.F.Smith and J.K.Scott, Methods in Enzymology, 217H, 228-257,1993) or *in vivo* (R.Pasqualini & E.Ruoslahti, Nature, 380, 364-366, 1996) are suitable elements for use in the invention.

Sequences derived from fibronectin or vitronectin binding proteins and from the complementarity-determining regions of monoclonal antibodies raised against epitopes within membrane proteins (see, for example, J.W.Smith *et al.* J.Biol.Chem. 270, 30486-30490, 1995) are also suitable binding or targeting elements.

Associations of heterologous amino acid sequences with a polypeptide which is a soluble derivative of a human protein will need to be assessed for potential immunogenicity, particularly where the amino acid sequence is not derived from a human protein and where repeated administration is envisaged. In general, this issue can be addressed by using sequences as close as possible to known human ones and through computation of secondary structure and antigenicity indices.

The polypeptide portion of the derivatives of the invention may be prepared by expression in suitable hosts of modified genes encoding the soluble polypeptide of interest plus one or more peptidic membrane binding elements and optional residues such as cysteine to introduce linking groups to facilitate post translational derivatisation with additional

membrane binding elements.

In a further aspect, therefore, the invention provides a process for preparing a derivative according to the invention which process comprises expressing DNA encoding the polypeptide portion of said derivative in a recombinant host cell and recovering the product and thereafter post translationally modifying the polypeptide to introduce membrane binding elements chemically.

In particular, the recombinant aspect of the process may comprise the steps of:

- i) preparing a replicable expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said polypeptide portion;
- ii) introducing an expression vector into the host cell;
- iii) culturing said host cell under conditions permitting expression of said DNA polymer to produce said polypeptide; and
- iv) recovering said polypeptide.

Where the polypeptide portion is novel, the DNA polymer comprising a nucleotide sequence that encodes the polypeptide portion as well as the polypeptide portion itself and S-derivatives thereof, also form part of the invention. In particular the invention provides a polypeptide portion of a derivative of the invention comprising the soluble peptide linked by a peptide bond to one peptidic membrane binding element and/or including a C-terminal cysteine, and DNA polymers encoding the polypeptide portion.

The recombinant process of the invention may be performed by conventional recombinant techniques such as described in Sambrook *et al.*, Molecular Cloning : A laboratory manual 2nd Edition. Cold Spring Harbor Laboratory Press (1989) and DNA Cloning vols I, II and III (D. M. Glover ed., IRL Press Ltd).

The invention also provides a process for preparing the DNA polymer by the condensation of appropriate mono-, di- or oligomeric nucleotide units.

The preparation may be carried out chemically, enzymatically, or by a combination of the two methods, *in vitro* or *in vivo* as appropriate. Thus, the DNA polymer may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts *et al.*, in Biochemistry 1985, 24, 5090-5098.

The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by enzymatic polymerisation, or by a combination of these methods.

Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) or the Taq or Pfu polymerases in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-72°C, generally in a volume of 50µl or less.

Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to 37°C, generally in a volume of 50µl or less.

The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J.Gait, H.W.D. Matthes M. Singh, B.S. Sproat and R.C. Titmas, *Nucleic Acids Research*, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, *Tetrahedron Letters*, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, *Tetrahedron Letters*, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, *Journal of the American Chemical Society*, 1981, 103, 3185; S.P. Adams *et al.*, *Journal of the American Chemical Society*, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus and H. Koester, *Nucleic Acids Research*, 1984, 12, 4539; and H.W.D. Matthes *et al.*, *EMBO Journal*, 1984, 3, 801. Preferably an automated DNA synthesiser (for example, Applied Biosystems 381A Synthesiser) is employed.

The DNA polymer is preferably prepared by ligating two or more DNA molecules which together comprise a DNA sequence encoding the polypeptide.

The DNA molecules may be obtained by the digestion with suitable restriction enzymes of vectors carrying the required coding sequences.

The precise structure of the DNA molecules and the way in which they are obtained depends upon the structure of the desired product. The design of a suitable strategy for the construction of the DNA molecule coding for the polypeptide is a routine matter for the skilled worker in the art.

In particular, consideration may be given to the codon usage of the particular host cell. The codons may be optimised for high level expression in *E. coli* using the principles

set out in Devereux *et al.*, (1984) Nucl. Acid Res., 12, 387.

The expression of the DNA polymer encoding the polypeptide in a recombinant host cell may be carried out by means of a replicable expression vector capable, in the host cell, of expressing the DNA polymer. Novel expression vectors also form part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment, encode the polypeptide, under ligating conditions.

The ligation of the linear segment and more than one DNA molecule may be carried out simultaneously or sequentially as desired.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired. The choice of vector will be determined in part by the host cell, which may be prokaryotic, such as *E. coli*, or eukaryotic, such as mouse C127, mouse myeloma, chinese hamster ovary, fungi e.g. filamentous fungi or unicellular 'yeast' or an insect cell such as *Drosophila melanogaster*, or *Spodoptera frugiperda*. The host cell may also be in a transgenic animal. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses derived from, for example, baculoviruses or vaccinia.

The DNA polymer may be assembled into vectors designed for isolation of stable transformed mammalian cell lines expressing the fragment e.g. bovine papillomavirus vectors in mouse C127 cells; or amplified vectors in chinese hamster ovary cells (DNA Cloning Vol. II D.M. Glover ed. IRL Press 1985; Kaufman, R.J. *et al.*. Molecular and Cellular Biology 5, 1750-1759, 1985; Pavlakis G.N. and Hamer, D.H. Proceedings of the National Academy of Sciences (USA) 80, 397-401, 1983; Goeddel, D.V. *et al.*, European Patent Application No. 0093619, 1983).

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Sambrook *et al.*, cited above. Polymerisation and ligation may be performed as described above for the preparation of the DNA polymer. Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under

transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Sambrook *et al.*, cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E. coli*, may be treated with a solution of CaCl_2 (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl , MnCl_2 , potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol or by electroporation as for example described by Bio-Rad Laboratories, Richmond, California, USA, manufacturers of an electroporator. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells or by using cationic liposomes.

The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Sambrook *et al.*, and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The protein product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial such as *E. coli* and the protein is expressed intracellularly, it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is eukaryotic, the product is usually isolated from the nutrient medium.

Where the host cell is bacterial, such as *E. coli*, the product obtained from the culture may require folding for optimum functional activity. This is most likely if the protein is expressed as inclusion bodies. There are a number of aspects of the isolation and folding process that are regarded as important. In particular, the polypeptide is preferably partially purified before folding, in order to minimise formation of aggregates with contaminating proteins and minimise misfolding of the polypeptide. Thus, the removal of contaminating *E. coli* proteins by specifically isolating the inclusion bodies and the subsequent additional purification prior to folding are important aspects of the procedure.

The folding process is carried out in such a way as to minimise aggregation of intermediate-folded states of the polypeptide. Thus, careful consideration needs to be given

6M guanidine hydrochloride. The next step is to remove these agents to allow folding of the proteins to occur. Formation of the disulphide bridges requires an oxidising environment and this may be provided in a number of ways, for example by air, or by incorporating a suitable redox system, for example a mixture of reduced and oxidised glutathione.

Preferably, the inclusion body is solubilised using 8M urea, in the presence of mercaptoethanol, and protein is folded, after initial removal of contaminating proteins, by addition of cold buffer. Suitable buffers may be identified using the techniques described in I.Dodd *et al*, 'Perspectives in Protein Engineering and Complementary Technologies', Mayflower Publications, 66-69, 1995. A suitable buffer for many proteins is 20 - 300mM ethanolamine containing 1mM reduced glutathione and 0.5mM oxidised glutathione. The folding is preferably carried out at a temperature in the range 1 to 50°C over a period of 1 to 4 days.

If any precipitation or aggregation is observed, the aggregated protein can be removed in a number of ways, for example by centrifugation or by treatment with precipitants such as ammonium sulphate. Where either of these procedures are adopted, monomeric polypeptide is the major soluble product. If the bacterial cell secretes the protein, folding is not usually necessary.

The polypeptide portion of the derivative of the invention may include an unpaired (preferably C-terminal) cysteine to facilitate post-translational modification. A soluble anti-angiogenic polypeptide including a unique free and preferably C-terminal cysteine also forms part of the invention.

Expression in a bacterial system is preferred for proteins of moderate size (up to ~70kDa) and with <~8 disulphide bridges. More complex proteins for which a free terminal cysteine could cause refolding or stability problems may require stable expression in eukaryotic cell lines (especially CHO). This will also be needed if a carbohydrate

membrane-binding element is to be introduced post-translationally. The use of insect cells infected with recombinant baculovirus encoding the polypeptide portion is also a useful general method for preparing more complex proteins and will be preferred when it is desired to carry out certain post-translational processes (such as palmitoylation) biosynthetically (see for example, M.J.Page *et al* J.Biol.Chem. 264, 19147-19154, 1989).

Specific methods for the expression and isolation of recombinant kringle domains are disclosed in for example, S.Cleary *et al*, Biochemistry. 28, 1884-1891, 1989, V.S.DeSerrano *et al*, Arch.Biochem.Biophys. 294, 282-290, 1992, N. Menhart *et al*, Biochemistry. 30, 1948-1957, 1991 and D.Marti *et al*, Eur.J.Biochem. 219, 455-462, 1994.

A preferred method of handling proteins C-terminally derivatised with cysteine is as a mixed disulphide with mercaptoethanol or glutathione or as the 2-nitro, 5-carboxyphenyl thio- derivative as generally described below in Methods.

Peptide membrane binding elements may be prepared using standard solid state synthesis such as the Merrifield method and this method can be adapted to incorporate required non-peptide membrane binding elements such as N-acyl groups derived from myristic or palmitic acids at the N terminus of the peptide. In addition activation of an amino acid residue for subsequent linkage to a protein can be achieved during chemical synthesis of such membrane binding elements. Examples of such activations include formation of the mixed 2-pyridyl disulphide with a cysteine thiol or incorporation of an N-haloacetyl group. Both of these groups are capable of reaction with free thiols, through disulphide interchange and alkylation, respectively. Peptides can optionally be prepared as the C-terminal amide and/or with a conventional N-terminal blocking group such as acetyl.

The invention also provides a peptidic membrane binding element derivatised as disclosed in WO98/02454 and preferably having one or more of the following features:

- a terminal cysteine residue optionally activated at the thiol group;
- an N-haloacetyl group (where halo signifies chlorine, bromine or iodine) located at the N-terminus of the the peptide or at an ϵ -amino group of a lysine residue;
- an amide group at the C-terminus;
- an N-terminal blocking group; and
- a fatty acid N-acyl group at the N-terminus or at an ϵ -amino group of a lysine residue.

Chemical bridging groups, reagents suitable for their formation and conditions for

their reaction with proteins include those described in WO98/02454. 2-iminothiolane is especially useful as a protein attachment group for the present invention. A preferred method for creating a free thiol function as a protein attachment group involves partial reduction of a disulphide-containing protein with a reducing agent. Preferably, the protein is derived from the N-terminal 561 aminoacids of human plasminogen and the reducing agent is tris-(2-carboxyethyl) phosphine.

The polypeptides to be linked are reacted separately with the linking agent or the reagent for introducing a protein attachment group by typically adding an excess of the reagent to the polypeptide, usually in a neutral or moderately alkaline buffer, and after reaction removing low molecular weight materials by gel filtration or dialysis. The precise conditions of pH, temperature, buffer and reaction time will depend on the nature of the reagent used and the polypeptide to be modified. The polypeptide linkage reaction is preferably carried out by mixing the modified polypeptides in neutral buffer in an equimolar ratio. Other reaction conditions e.g. time and temperature, should be chosen to obtain the desired degree of linkage. If thiol exchange reactions are involved, the reaction should preferably be carried out under an atmosphere of nitrogen. Preferably, UV-active products are produced (eg from the release of pyridine 2-thione from 2-pyridyl dithio derivatives) so that coupling can be monitored.

After the linkage reaction, the polypeptide conjugate can be isolated by a number of chromatographic procedures such as gel filtration, ion-exchange chromatography, affinity chromatography or hydrophobic interaction chromatography (HIC). These procedures may be either low pressure or high performance variants. Convenient generic final stage purification strategies are HIC on C2-C8, preferably C4, media and cation exchange chromatography. These methods are preferred for separation of derivatised and underivatised proteins into which a hydrophobic-electrostatic switch combination has been inserted. Affinity chromatography on immobilised Lysine or 6-aminohexyl (AH) media is a preferred technique for isolation of plasminogen derivatives containing lysine or AH binding sites.

The conjugate may be characterised by a number of techniques including low pressure or high performance gel filtration, SDS polyacrylamide gel electrophoresis, isoelectric focussing or electrospray mass spectrometry.

In one preferred aspect the present invention relates to derivatives of human plasminogen kringle domains linked through one or more disulphide bridges to:

N-myristoyl AspGlyProLysLysLysLysLysLysSerProSerLysSerSerGlyCys
 N-myristoyl GlySerSerLysSerProSerLysLysLysLysLysLysProGlyAspCys
 CysAspGlyProLysLysLysLysLysLysSerProSerLysSerSerLys(N-ε-myristoyl)
 N-myristoyl SerLysAspGlyLysLysLysLysLysLysSerLysThrLysCys
 [SEQ ID Nos. 17-20]

A further preferred aspect incorporates a C-terminal extension to the above proteins. For example the sequence: AspGlyProSerGluLeuArgGlyAspPheSerSerCys [SEQ ID No. 21] or AlaSerAspAlaArgGlyAspSerPheAlaGlyCys [SEQ ID No. 22] linked to any of the above membrane-targeting derivatives optionally with additional spacer sequences immediately preceding the cysteine of the protein extension.

The derivatives of this invention are preferably administered as pharmaceutical compositions.

Accordingly, the present invention also provides a pharmaceutical composition comprising a derivative of the invention in combination with a pharmaceutically acceptable carrier.

The compositions according to the invention may be formulated in accordance with routine procedures for administration by any route, particularly parenteral, especially by intravenous infusion but also including the oral, sublingual, subcutaneous, intraperitoneal and transdermal routes or by inhalation. The compositions may be in the form of tablets, capsules, powders, granules, lozenges, creams or liquid preparations, such as oral or sterile parenteral solutions or suspensions or in the form of a spray, aerosol or other conventional method for inhalation.

The topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions and eye or ear drops, impregnated dressings and aerosols and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams.

The formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

compound and a sterile vehicle, water being preferred. The compound, depending on the vehicle and concentration used, is dissolved in the vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilised before filling into a suitable vial or ampoule and sealing.

Parenteral formulations may include sustained-release systems such as encapsulation within microspheres of biodegradable polymers such as poly-lactic co-glycolic acid.

Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be supplied to reconstitute the liquid prior to use. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the compound.

Compositions of this invention may also suitably be presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns for example diameters in the range of 1-50 microns, 1-10 microns or 1-5 microns. Where appropriate, small amounts of anti-asthmatics and bronchodilators, for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and

aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included.

Microfine powder formulations may suitably be administered in an aerosol as a metered dose or by means of a suitable breath-activated device or transdermally by ballistic techniques.

Suitable metered dose aerosol formulations comprise conventional propellants, cosolvents, such as ethanol, surfactants such as oleyl alcohol, lubricants such as oleyl alcohol, desiccants such as calcium sulphate and density modifiers such as sodium chloride.

Suitable solutions for a nebulizer are isotonic sterilised solutions, optionally buffered, at for example between pH 4-7, containing up to 20mg ml⁻¹ of compound but more generally 0.1 to 10mg ml⁻¹, for use with standard nebulisation equipment.

The quantity of material administered will depend upon the potency of the derivative and the nature of the complaint be decided according to the circumstances by the physician supervising treatment. However, in general, an effective amount of the polypeptide for the treatment of a disease or disorder is in the dose range of 0.01-100mg/kg per day, preferably 0.1mg-10mg/kg per day, administered in up to five doses or by infusion.

No adverse toxicological effects are indicated with the compounds of the invention within the above described dosage range.

The invention also provides a derivative of the invention for use as a medicament.

The invention further provides a method of treatment of disorders amenable to treatment by a soluble peptide which comprises administering a soluble derivative of said soluble peptide according to the invention, and the use of a derivative of the invention for the preparation of a medicament for treatment of such disorders.

The present invention is also directed to a pharmaceutical composition for treating an established primary tumour and for preventing the growth of secondary tumours following surgery.

The following Methods and Examples illustrate the invention.

GENERAL METHODS USED IN EXAMPLES

(i) Amplification of DNA by the Polymerase Chain Reaction

The polymerase chain reaction was used to amplify a segment of DNA from template

manufacturer's guidelines.

(vii) Introduction of DNA into *E. coli* and selection of recombinant clones

Plasmid DNA was used to transform competent *E. coli* cells (typically strain XL1 Blue - Stratagene) according to the supplier's instructions. Transformed cells were selected by their ability to grow on media containing appropriate antibiotic, typically 100 µg/ml ampicillin.

(viii) DNA sequencing

DNA sequencing was performed using automated fluorescent DNA sequencing under contract (Lark Technologies, Inc.).

(ix) Production of oligonucleotides

Oligonucleotides were purchased from Genosys.

(x) Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the Novex system (Novex GmbH, Heidleberg, Germany) according to the manufacturer's instructions. Pre-packed gels containing a 4- 20% acrylamide gradient were used. Samples for electrophoresis, including protein molecular weight standards (for example LMW Kit, Pharmacia, Sweden or Novex Mark 12, Novex, Germany) were usually diluted 1:1 in 2% (w/v) SDS - containing buffer (with or without 5% (v/v) 2-mercaptoethanol), and left at room temperature for 0.5h before application to the gel.

(xi) Reduction of disulphides and modification of thiols in proteins

Selective reduction of disulphide bonds may be necessary because, during the isolation and purification of multi-thiol proteins (in particular those containing terminal cysteine and internal thiol pairs), inappropriate disulphide pairing can occur during the refolding of a fully denatured protein. In addition, even if correct disulphide pairing does occur, it is possible that a free cysteine in the protein may become blocked, for example with glutathione. These derivatives are generally quite stable. In order to make them more reactive, for example for subsequent conjugation to another functional group, they need to be selectively reduced, with for example dithiothreitol (DTT) or with Tris (2-carboxyethyl) phosphine.HCl (TCEP), then

either used directly or modified to give a moderately activated disulphide derivative. Ellman's reagent (DTNB) is an example of a reagent, which gives such mixed disulphides with free thiols. In the case where treatment with DTNB is omitted, careful attention to experimental design is necessary to ensure that dimerisation of the free thiol-containing protein is minimised. Reference to the term 'selectively reduced' above means reaction conditions eg. duration, temperature and molar ratios of reactants have to be carefully controlled so that only the disulphide bridge at the C-terminus of the protein is reduced or that reduction of only the desired disulphide within the natural architecture of the protein is achieved. TCEP is available from Pierce & Warriner (Chester, Cheshire) and the following general example illustrates the type of conditions that may be used and that are useful for the generation of free thiols and their optional modification.

TCEP may be prepared as a 20mM solution in 50mM HEPES (approx. pH 4.5) and may be stored at -40°C. DTT may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40°C. DTNB may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40°C. All of the above reagents are typically used at molar equivalence or molar excess, the precise concentrations ideally identified experimentally. The duration and the temperature of the reaction are similarly determined experimentally. Generally the duration would be in the range 1 to 24 hours and the temperature would be in the range 2 to 30°C. Excess reagent may be conveniently removed by buffer exchange, for example using Sephadex G25. A suitable buffer is 0.1M sodium phosphate pH 7.0.

EXAMPLES

Example 1: Preparation of a conjugate of human plasminogen kringles 1-3 with Myristoyl Switch Peptide 1 (MSWP-1).

The K1-K3 fragment of human plasminogen (also known as lysine binding site 1) was prepared by the method of L. Sottrup-Jensen et al, 1978, *loc cit* and used as a solution 1.7mg/ml (~50micromolar) in 0.1M sodium phosphate buffer pH 7.0.

To this solution (50 µl) was added either 1 µl or 2.5 µl of a solution of 5mM TCEP in 50 mM

HEPES pH 7.4 corresponding to final concentrations of 100 μ M and 250 μ M respectively. Aliquots were incubated in sealed microtubes tubes at 16 °C for 14h to effect partial reduction of the kringle disulphide bonds. Myristoyl switch peptide 1 (Example 2 of WO98/02454, 2.5 μ l of a 10mM solution in 0.1M sodium chloride, 0.05M sodium phosphate pH 7.2) was added to a final concentration of approximately 0.5mM (10-fold molar excess over protein). The mixture was incubated at 16°C for 15 min and then held at 4 °C for 2h before being frozen at -70°C. SDS-PAGE analysis of these materials was performed in comparison with a control to which MSWP-1 but not TCEP had been added. Control K1-K3 showed three main bands at apparent molecular weights of 29kDa, 34kDa and 37kDa and weak bands at higher molecular weight corresponding to the components of 'Pool II' described by Sottrup-Jensen et al. Pretreatment with TCEP in 2 or 5-fold molar excess resulted in a shift of the main triplet to give new bands with apparent Mr 38kDa, 43kDa and 47kDa. If higher molar ratios of TCEP were used, bands became indistinct and a discrete gel shift was not observed.

The reaction mixture (0.05ml) of K1-K3-MSWP1 described above was diluted with 0.1M sodium phosphate pH7.0 (0.05ml) and mixed with Toyopearl Butyl 650M matrix (80mg) that had been washed in 0.1M sodium phosphate pH7.0 and suction-dried. The suspension was mixed for 5 min at 4 degrees C, then centrifuged at 10000g for 2 min. The supernatant was removed and retained. The pellet (matrix) was washed with 2 x 0.4ml of 0.1M sodium phosphate pH7.0 and adsorbed protein was then eluted using 0.1ml of 0.6M ethanolamine (to give a final ethanolamine concentration of about 0.3M). Analysis of all the relevant fractions by SDS PAGE (Novex, 4-20% acrylamide gradient), using Mk12 standards (Novex), followed by staining with Coomassie Brilliant Blue R250, showed that two species, a ~ 40K putative K1-K3-MSWP1 species and another polypeptide at apparent molecular weight approx. 50K, had adsorbed to the matrix and had been eluted with the ethanolamine buffer. These results provide evidence for the successful conjugation of MSWP1 and K1-K3, as the primary interaction between K1-K3-MSWP1 and the Toyopearl Butyl matrix is via the MSWP1 moiety.

The results suggest that between 2 and 4 molecules of MSWP-1 were added per mole of K1-K3.

Example 2: Preparation of K78-R561 plasminogen (C558-[MSWP1])

The preparation of the title compound is carried out in stages.

- (i) Involves isolation of the plasmin A chain (residues K78-R561 in the amended numbering of M.Fordgren et al, FEBS Letters, 213, 254-260, 1987) with a free thiol at the C558 position using methodology similar to that described in US 4,908,204. The method involves reduction of the interchain disulphide with 50mM DTT under appropriate buffer conditions, followed by separation of the A-chain from the B-chain using chromatography on lysine-Sepharose. Most importantly, the method of isolation is capable of generating an A-chain preparation with a free thiol at the C558 position.
- (ii) The plasmin A-chain and MSWP-1 molecules are mixed under appropriate conditions to generate the title compound. Appropriate conditions are described in more detail in WO 98/02454 and include, for example, reaction for a short period of time (2 to 4h) in 0.1M sodium phosphate pH 8.0 at 20°C of a small molar excess of MSWP-1 over plasmin A-chain. Typically, 0.05mM plasmin A-chain and 0.2mM MSWP-1 would be used. The product of the invention is identified in the first instance by the detection of a small decrease in mobility of the modified plasmin A-chain (compared to unmodified) on SDS PAGE, corresponding to the addition of the approx. 2K ligand.
- (iii) The product of the invention is purified to homogeneity, using for example, hydrophobic interaction chromatography or affinity chromatography on Lysine-Agarose, and formulated for use as a clinically acceptable preparation by buffer-exchange into a suitable buffer, optionally in the presence of excipients such as stabilisers (eg. Mannitol), and optionally lyophilised.

Example 3: Production of kringle 5, comprising human plasminogen (S458-C548), using an *E.coli* expression system, and its conjugation to MSWP-1.

The following stages are involved in the preparation of the title compound:

- (i) Preparation of DNA encoding kringle 5

- (ii) Expression vector construction for *E.coli* expression
- (iii) Expression, refolding & isolation of kringle 5 from *E.coli*
- (iv) Modification with membrane binding elements

i) Preparation of DNA encoding kringle 5

The DNA sequence encoding kringle 5 of human plasminogen is contained for example within the plasmids encoding the hybrid plasminogen activators (eg. pDB850) described in US5302390. The desired sequence of the mature polypeptide is given in sequence ID number 1:

Sequence ID No 1

SerGluGluAspCysMetPheGlyAsnGlyLysGlyTyrArgGlyLysArgAlaThrThr
ValThrGlyThrProCysGlnAspTrpAlaAlaGlnGluProHisArgHisSerIlePheThr
ProGluThrAsnProArgAlaGlyLeuGluLysAsnTyrCysArgAsnProAspGlyAsp
ValGlyGlyProTrpCysTyrThrThrAsnProArgLysLeuTyrAspTyrCysAspVal
ProGlnCysAlaAlaProSerPheAspCys

The kringle 5 DNA was amplified from the vector pDB850 using the polymerase chain reaction and a pair of oligonucleotide primers partially complementary to the kringle 5 sequence, but containing additional nucleotides to introduce additional amino acid residues or restriction sites as follows:

Primer 1 (Sequence ID No 2) introduces a restriction site for *NdeI*, a site that is present in the *E. coli* vector pBroc413, and also introduces a methionine residue, for translation initiation, at the N-terminus of the desired sequence.

Primer 2 (Sequence ID Number 3) introduces the additional amino acid sequence Ala-Ala-Pro-Ser-Phe-Asp-Cys [SEQ ID No. 23] at the C-terminus of the polypeptide. This sequence is native to plasminogen, but does not appear in some of the hybrid plasminogen activator plasmids. The terminal cysteine is required for subsequent chemical modification. In addition to the amino acids, a translation termination codon and a BamHI site are added.

PCR Product 1 was purified and ligated directly into a holding vector, pCR2.1 (Invitrogen), and used to transform *E. coli* XL1 Blue cells. Plasmid DNA was prepared from several of the colonies of transformed cells and the integrity of the cloned PCR Product 1 in each determined by DNA sequencing. Plasmid carrying PCR Product 1, with the correct DNA sequence, was digested with restriction enzymes Nde I and BamH I to generate Fragment 1. The vector pBroc413 was digested with restriction enzymes Nde I and BamH I to generate Fragment 2. Fragments 1 & 2 were separated from by-products of the digestion reactions by agarose gel electrophoresis, then excised from the gel and purified. Purified Fragments 1 and 2 were ligated to generate vector pBC87-01.

(iii) Expression, refolding and isolation of kringle 5 from *E. coli*

pBC87-01 is used to transform competent *E. coli* cells (typically strain BL21(DE3) - Stratagene) under conditions recommended by the manufacturer. A single colony of freshly transformed cells is used to inoculate 5 ml of Luria Broth (Miller's Modification) (Sigma) containing 100ug/ml ampicillin, and grown at 37°C overnight. This is used to inoculate 500ml of the same medium in a 2 litre conical flask and incubated at 37°C, shaking at 200 rpm, until an optical density at 600nm of approximately 0.5 is reached. IPTG is added to a final concentration of 1mM and the cultures are shaken at 200 rpm for a further three hours. The cells are pelleted by centrifugation at 6000 rpm and the kringle 5 product refolded and recovered from inclusion bodies essentially as described by Wilhem O.G et al, (J.Biol.Chem, 265, 14606-14611, 1990) with the exception that 6-aminohexyl-Sepharose or 4-

aminobenzamidine-Sepharose are employed to purify the refolded kringle.

(iv) Modification with membrane binding elements

Kringle 5 products are first reduced using TCEP and then reacted with MSWP-1 as described in Example 2. The modified product is isolated by ultrafiltration using 10kDa cut-off membranes or by hydrophobic interaction chromatography on Toyopearl Butyl as described in Example 1.

Example 4: Production of kringle 5, comprising human plasminogen (S458-C548), using the Baculovirus expression system, and its conjugation to MSWP-1.

The following stages are involved in the preparation of the title compound:

- (i) Preparation of DNA encoding kringle 5
- (ii) Expression vector construction for baculovirus/insect cell expression
- (iii) Expression and isolation of kringle 5 from a baculovirus/insect cell system
- (iv) Modification with membrane binding elements

i) Preparation of DNA encoding kringle 5

The DNA sequence encoding kringle 5 of human plasminogen is contained, for example, within the plasmids encoding the hybrid plasminogen activators (eg. pDB850) described in US5302390. The desired sequence of the mature polypeptide is given in sequence ID number 1 (see Example 3). Using baculovirus vectors, the desired polypeptide sequence is expressed in insect cells preceded by a signal peptide to enable the mature polypeptide to be secreted into the culture medium. The signal peptide is derived from the baculovirus gene encoding gp64 and is attached to the kringle 5 coding region by cloning of the amplified kringle 5 DNA fragment into the expression vector pBacSurf-1 (Novagen).

The kringle 5 DNA was amplified from the vector pDB850 using the polymerase chain reaction and a pair of oligonucleotide primers partially complementary to the kringle 5 sequence, but containing additional nucleotides to introduce additional amino acid residues or

Primer 3 (sequence ID No 4) introduces an Nsi I site, which, upon cleavage, creates a cohesive end compatible with the Pst I site in the baculovirus vector, pBacSurf-1.

Primer 4 (sequence ID Number 5) introduces the additional amino acid sequence Ala-Ala-Pro-Ser-Phe-Asp-Cys at the C-terminus of the polypeptide. This sequence is native to plasminogen, but does not appear in some of the hybrid plasminogen activator plasmids. The terminal cysteine is required for subsequent chemical modification. In addition to the amino acids, a translation termination codon and a BamH I site are added.

The sequence encoding kringle 5 was amplified using Primer 3 and Primer 4 (Seq ID No 4 and 5) to generate PCR Product 2.

Sequence ID No. 4: Primer 3

GGATGCATTCCGAAGAAGACTGTATGTTTGGGAAT

Sequence ID No. 5: Primer 4

GGGGATCCTTAGCAGTCGAAGGACGGAGCAGCACACTGAGGGACATCACAGTAG
TCGT

(ii) Expression vector construction for baculovirus/insect cell expression

PCR Product 2 was purified and ligated directly into a holding vector, pCR2.1 (Invitrogen), and used to transform *E.coli* XL1 Blue cells. Plasmid DNA was prepared from several of the colonies of transformed cells and the integrity of the cloned PCR Product 2 in each determined by DNA sequencing. Plasmid carrying PCR Product 2, with the correct DNA sequence, was digested with restriction enzymes Nsi I and BamH I to generate Fragment 3. The vector pBacSurf-1 was digested with enzymes Pst I and BamH I to generate Fragment 4. Fragments 3 and 4 were separated from by-products of the digestion reactions by agarose gel electrophoresis, then excised from the gel and purified. Purified Fragments 3 and 4 were ligated to generate vector pAS87-01.

(iii) Expression and isolation of kringle 5 from a baculovirus/insect cell system

pAS87-01 is used to generate the baculovirus expression vector vAS87-01 by cotransfection with linearised baculovirus DNA into Sf9 cells using components of the BacPak kit (Clontech) under conditions recommended by the manufacturer. Recombinant baculoviruses containing the kringle 5 gene are identified by Western blot or activity assay of the recombinant protein, which is present in the culture supernatant. A single confirmed clone of vAS87-01 is plaque purified and scaled up.

Sf9 cells are grown at 28°C under low serum or serum-free conditions in IPL41 medium (Sigma), with supplements of yeast hydrolysate (Sigma), at a dilution of 1 in 50, Lipid mixture (Sigma), at a dilution of 1 in 1000, and Pluronic (10% stock) (Sigma), at a dilution of 1 in 100. Cells are grown to a density of between 5×10^5 and 1×10^6 cells/ml and infected with vAS87-01 at a multiplicity of infection (MOI) of 5 plaque-forming units (pfu) per cell. The infected cultures are grown for a further 48-72 hours prior to harvesting the supernatant. The kringle 5 product is isolated directly by chromatography on 6-aminohexyl- or 4-aminobenzamidine-Sepharose.

(iv) Modification with membrane binding elements

Kringle 5 products are first reduced using TCEP and then reacted with MSWP-1 as described in Example 2. The modified product is isolated by ultrafiltration using 10kDa cut-off membranes or by hydrophobic interaction chromatography on Toyopearl Butyl as described in Example 1.

Example 5: Production of kringle 5, comprising human plasminogen (S458-C548), using the *Pichia pastoris* expression system, and its conjugation to MSWP-1.

The following stages are involved in the preparation of the title compound

i) Preparation of a *Pichia pastoris* shuttle vector

- ii) Preparation of DNA encoding kringle 5
- iii) Expression vector construction for the *Pichia pastoris* expression
- iv) Expression & isolation of kringle-5 from *Pichia pastoris*
- v) Modification with membrane binding elements

(i) Preparation of a *Pichia pastoris* shuttle vector

The *Pichia pastoris* expression vector used to express human kringle 5 was pPIC9K. This vector contains the *Saccharomyces cerevisiae* α -factor mating pheromone pre-peptide signal sequence, which, when fused to the N-terminus of a foreign protein, will direct its secretion from the host cell; this signal sequence is adjacent to the vector's multiple-cloning site. A Xho I restriction site, convenient for the introduction of foreign DNA, lies shortly before the multiple cloning site. However, a second Xho I site lies elsewhere in pPIC9K and digestion of the vector to cleave the first Xho I site selectively is inefficient. To surmount this problem, a portion of pPIC9K, encoding the signal sequence and the multiple-cloning site, was subcloned in a different vector, to generate a shuttle vector in which the 'convenient' Xho I site is unique.

The vector pPIC9K was digested with restriction enzymes BamH I and Not I to release Fragment 5, encoding the signal sequence and the vector's multiple-cloning site. The digested DNA was subjected to agarose gel electrophoresis, Fragment 5 excised and purified.

A commercially available vector, pUC19 (New England Biolabs), containing a unique BamH I site but no Not I site, was subjected to site-directed mutagenesis to introduce a unique Not I site near the BamH I site. Mutagenesis was performed using the two complementary mutagenic oligonucleotides, Primer 5 (Sequence ID No 6) and Primer 6 (Sequence ID No 7):

Sequence ID No. 6: Primer 5

CGAGCTCGAATTCAGTGGCGGCCGCTTTACAACGTCGTGACTGG

Sequence ID No. 7: Primer 6

CCAGTCACGACGTTGTAAAGCGGCCCGCCAGTGAATTCGAGCTCG

The vector generated by the mutagenesis reaction, pUC19N, was digested

simultaneously with restriction enzymes BamH I and Not I and purified using a Size-Sep column (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Purified Fragment and BamH I / Not I digested pUC19N were ligated to generate the *Pichia pastoris* shuttle vector pUCPIC, which was used to transform competent *E.coli* XL1 Blue cells (Stratagene). A single colony of transformed cells was used to inoculate 50 ml LB medium containing 100 µg/ml ampicillin, grown overnight at 37°C, shaking at 200 rpm, and used to prepare pUCPIC plasmid DNA.

(ii) Preparation of DNA encoding kringle 5

The desired sequence of the mature kringle 5 polypeptide is given in sequence ID No 1 (see Example 3). Kringle 5 DNA was amplified from the vector pBC87-01 (See Example 3), using the polymerase chain reaction and a pair of oligonucleotide primers partially complementary to the kringle 5 sequence, but containing additional nucleotides to add additional amino acid residues or restriction sites as follows:

Primer 7 (Sequence ID No 8) was designed to introduce a unique Xho I restriction site and to encode a Lys-Arg dipeptide contiguous with and upstream of the region encoding kringle 5. This dipeptide is necessary to ensure that the signal sequence is cleaved from the mature kringle 5 polypeptide before its secretion into the culture medium.

Primer 8 (Sequence ID No 9) was designed to introduce a unique EcoRI restriction site and to introduce a translation termination codon, contiguous with and downstream of the C-terminal cysteine residue of kringle 5.

Sequence ID No. 8: Primer 7

GGGTATCTCTCGAGAAAAGATCCGAAGAAGACTGTATGTTTG

Sequence ID No. 9: Primer 8

GCCCTAGGGAATTCAGCAGTCGAAGGACGGAGC

The sequence encoding kringle 5 was amplified from pBC87-01 using the polymerase chain

reaction and Primers 7 and 8 (Sequence ID Nos 8 and 9) to generate PCR Product 3.

(iii) Expression vector construction for the *Pichia pastoris* expression

PCR Product 3 was purified and digested using restriction enzymes XhoI and EcoRI to generate Fragment 6. The digested DNA was subjected to agarose gel electrophoresis, Fragment 6 excised and purified. Shuttle vector pUCPIC was digested using restriction enzymes XhoI and EcoRI and purified using a Size-Sep column (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Purified Fragment 6 and Xho I / EcoRI digested pUCPIC were ligated to generate the vector pUCPIC87-01, which was used to transform competent *E.coli* XL1 Blue cells (Stratagene).

The integrity of the DNA encoding kringle 5 in vector pUCPIC87-01, was verified by DNA sequencing (Lark Technologies, Inc.). The vector was digested with restriction enzymes BamH I and Not I to generate Fragment 7, which was subjected to agarose gel electrophoresis and purified. The vector pPIC9K was digested with restriction enzymes BamH I and Not I and purified using a Size-Sep column (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Purified Fragment 7 and BamH I / Not I digested pPIC9K were ligated to generate the vector pAG87-01, which was cloned in *E.coli* XL1 Blue cells (Stratagene).

(iv) Expression & isolation of kringle 5 from *Pichia pastoris*

Spheroplasts of *Pichia pastoris* cells (strain SMD1168 - Invitrogen) were prepared and transfected with 10 µg of purified Sal I-digested pAG87-01 according to the Multi-Copy *Pichia* Expression Kit Instruction Manual (Invitrogen). *Pichia pastoris* SMD1168 cells carry a mutation in their histidinol dehydrogenase gene, rendering them incapable of growth in the absence of the amino acid histidine. Cells transformed with the vector pAG87-01 were selected for their ability to grow on medium lacking histidine, because the vector carries a functional copy of the histidinol dehydrogenase gene.

Expression of kringle 5 from the transformed cells was examined using the experimental guidelines given in the Multi-Copy *Pichia* Expression Kit Instruction Manual. Expression of foreign proteins from *Pichia pastoris*, using vectors derived from pPIC9K, is accomplished in two stages: (i) a growth phase, typically using buffered minimal glycerol

(BMG) medium and (ii) an induction phase, typically using buffered minimal methanol (BMM) medium. The cells grow rapidly in BMG medium but no foreign protein can be produced, because the promoter controlling transcription of the foreign gene is switched off. The cells grow slowly in the BMM medium, but the promoter controlling transcription of the foreign gene is switched on. A typical experiment to screen for expression of a foreign protein uses the growth phase to accumulate biomass followed by an induction phase to accumulate foreign protein.

10 colonies of transformed cells were picked at random and used to inoculate 5ml BMG medium in 50 ml Falcon tubes. Cultures were incubated at 30°C, shaking at 250 rpm, for 36 hours, the cells pelleted by centrifugation at 1500g for 10 min, the supernatant discarded and the cells resuspended in 5 ml BMM medium (containing 1% v/v methanol). Cultures were incubated at 30°C, shaking at 250 rpm, for 48 hours, with addition of 1 % (v/v) methanol after 24 hours. Cells were pelleted by centrifugation and samples of culture supernatant analysed by SDS-PAGE for the presence of kringle 5. All 10 clones produced kringle 5, under these conditions, at an expression level of 1-2 mg soluble protein / litre of culture medium.

One clone was used to inoculate 30 ml of BMG medium and incubated at 30°C, shaking at 250 rpm, for 24 hours, to produce a starter culture for a large-scale induction experiment. Four 5 litre conical flasks, each containing 750 ml BMG, were each inoculated with 7.5 ml of the starter culture and incubated for 36 hours at 30°C, shaking at 200 rpm. The cells were harvested by centrifugation at 1500g for 10 min, the culture supernatant discarded and the cells resuspended in four 750 ml aliquots of BMM (containing 1% v/v methanol). The four aliquots were returned to the four 5 litre conical flasks and incubated for 60 hours at 30°C, shaking at 200 rpm; 1 % (v/v) methanol was added after 24 hours and after 48 hours. The cells were then removed by centrifugation at 1500g for 20 min, the culture supernatants pooled and stored at 4°C.

The pH of the pooled culture supernatant was adjusted to pH 3.0 using 5 M HCl, then the acidified supernatant was passed through a column of Macrorep Hi-S cation exchange matrix (BioRad), pre-equilibrated in 20mM acetic acid. The column was washed with 20mM sodium acetate buffer, pH 4.6 and the bound kringle 5 protein eluted by washing with 20mM sodium acetate buffer, pH 4.6, containing 1 M NaCl; the eluted fraction was regarded as the product. Analysis of the product by SDS PAGE followed by staining for protein revealed

of Kringle 5. Further purification of Kringle 5 is accomplished by chromatography on 6-aminohexyl- or 4-aminobenzamidine-Sepharose.

(v) Modification of cysteine-tailed kringle 5 with MSWP-1

The kringle 5 from the cation-exchange purification step was concentrated by ultrafiltration using a 3 kDa cut-off membrane in a stirred-cell (Amicon). A sample of material was buffer exchanged in 100 mM sodium phosphate buffer, pH 7.4 to generate a 180 μ M solution of kringle 5 (approx. 1.8 mg/ml). Three 200 μ l aliquots were taken and 10 mM TCEP added to each such that the final molar ratio of TCEP to kringle 5 was 1.5, 2.0 or 3.0. These reactions were incubated overnight at room temperature then brought to a final concentration of 50mM ethanolamine. Each reaction was divided into three samples for MSWP-1 treatment: one of these remained untreated and 10mM MSWP-1 was added to the other two such that the final molar ratio of MSWP-1 to kringle 5 was nil, 2.0 or 4.0, respectively. These reactions were incubated at room temperature for 2 hours and analysed by non-reducing SDS-PAGE followed by staining for protein. A new species with an apparent molecular weight of approx. 12000Da was noted in samples treated with MSWP-1 but not in those where MSWP-1 had been omitted. This was consistent with the successful conjugation of MSWP-1 and kringle 5 cys. The modified product is isolated by hydrophobic interaction chromatography on Toyopearl Butyl in a similar way to that described in Example 1.

Claims

1. A soluble derivative of a soluble anti-angiogenic polypeptide, said derivative comprising two or more heterologous membrane binding elements with low membrane affinity covalently associated with the polypeptide which elements are capable of interacting, independently and with thermodynamic additivity, with components of the vascular endothelium.
2. A derivative according to claim 1 wherein the soluble polypeptide is selected from the non-catalytic regions of human plasminogen (within the N-terminal 560 residues of that protein); fragments thereof, particularly those generated by metalloprotease digestion of plasminogen; fragments of related proteins containing kringle domains such as hepatocyte growth factor or apolipoprotein (a), prothrombin, tissue-type plasminogen activator, urinary-type plasminogen activator and hybrids thereof with plasminogen sequences; mutants of the above kringle domains, those containing positively charged to neutral or negatively charged mutations at positions 20, 21, 78 and 79 (numbering as used by Cao et al, 1997); fragments of collagen, particularly collagen XVIII; fragments of prolactin, the 16kDa N-terminal region of prolactin; neutralising antibodies against receptors for angiogenic mediators; antagonists of integrins involved in angiogenesis; and hybrids, derivatives or muteins thereof.
3. A derivative according to claim 1 or 2 wherein each membrane binding element with low membrane affinity has a dissociation constant of $1\mu\text{M}$ - 1mM .
4. A derivative according to any preceding claim wherein each membrane binding element has a size $<5\text{kDa}$.
5. A derivative according to any preceding claim which incorporates sufficient elements with low affinities for membrane components to result in a $0.01 - 10\text{nM}$ dissociation constant affinity for specific membranes.
6. A derivative according to any preceding claim which has a solubility in pharmaceutical formulation media $>100\mu\text{g/ml}$.
7. A derivative according to any preceding claim wherein at least one element is hydrophilic.
8. A derivative according to any preceding claim which comprises two to eight membrane binding elements.

9. A derivative according to any preceding claim wherein the membrane binding elements are selected from: fatty acid derivatives; basic amino acid sequences; ligands of known integral membrane proteins; sequences derived from the complementarity determining region of monoclonal antibodies raised against epitopes of membrane proteins; and membrane binding sequences identified through screening of random chemical or peptide libraries.
10. A derivative according to claim 9 wherein a membrane binding element is a fatty acid derivative selected from aliphatic acyl groups with about 8 to 18 methylene units, long-chain (8-18 methylene) aliphatic amines and thiols, steroid and farnesyl derivatives.
11. A derivative according to claim 9 or 10 wherein a membrane binding element is a basic amino acid sequence including (Lys)_n where n is from 3 to 10.
12. A derivative according to claim 11 wherein the binding elements are selected from:
 - i) DGPKKKKKKSPSKSSG
 - ii) GSSKSPSKKKKKKPGD
 - iii) SPSNETPKKKKKRFSFKKSG
 - iv) DGPKKKKKKSPSKSSK
 - v) SKDKKKKKKSKTK
 (N-terminus on left), [SEQ ID Nos. 10-14].
13. A derivative according to any of claims 9 to 11 wherein a membrane binding element is derived from a ligand of an integral membrane protein known to be associated with the vascular endothelium and angiogenic processes selected from the disulphide-constrained sequence ACDCRGDCFCG [SEQ ID No. 15]; DGPSEILRDFSS [SEQ ID No. 16] derived from human fibrinogen alpha chain, which binds to the GpIIb/IIIa membrane protein in platelets; sequences derived from fibronectin or vitronectin binding proteins and from the complementarity determining regions of monoclonal antibodies raised against epitopes within membrane proteins; $\alpha v\beta 3$ integrins or ligands having the RGD sequence.
14. A derivative according to any preceding claim which has the following structure:
 $[P]-\{L-[W]\}_n-X$
 in which: P is the soluble anti-angiogenic polypeptide, each L is independently an optional flexible linker group, each W is independently a peptidic membrane binding element, n is an integer of 1 or more and X is a peptidic or non-peptidic membrane-binding entity which may be covalently linked to any W.
15. A derivative according to claim 14 wherein peptidic membrane binding elements are 8 to 20 amino acids long, and elements W are located sequentially either at the N or C terminus of the soluble anti-angiogenic polypeptide and the amino acid sequences are

linked to one another and to the soluble anti-angiogenic peptide by linker groups which are selected from: hydrophilic and/or flexible amino acid sequences of 4 to 20 amino acids; linear hydrophilic synthetic polymers; and chemical bridging groups.

16. A derivative according to claim 14 or 15 wherein the chemical bridging groups are of formula (I):
 $-A-R-B-$
 in which each of A and B, which may be the same or different, represents $-CO-$, $-C(=NH_2^+)-$, maleimido, $-S-$ or a bond and R is a bond or a linking group containing one or more $-(CH_2)-$ or meta-, ortho- or para- disubstituted phenyl units optionally together with a hydrophilic portion.
17. A derivative according to claim 16 wherein R is selected from $-(CH_2)_r-$, $-(CH_2)_p-$, $-S-S-(CH_2)_q-$ and $-(CH_2)_p-CH(OH)-CH(OH)-(CH_2)_q-$, in which r is an integer of at least 2, and p and q are independently integers of at least 2, or $(CH_2)_2CONH(CH_2)_nNH-(4 \text{ phenyl})$ where n is an integer of 3 to 8.
18. A process for preparing a derivative according to any one of claims 1 to 17 which process comprises expressing DNA encoding the polypeptide portion of said derivative in a recombinant host cell and recovering the product and thereafter post translationally modifying the polypeptide to chemically introduce membrane binding elements.
19. A polypeptide portion of a derivative according to claim 1, comprising the soluble anti-angiogenic peptide linked by a peptide bond to one peptidic membrane binding element.
20. A soluble polypeptide according to claim 1 or 19 including a C-terminal cysteine.
21. A DNA polymer encoding the polypeptide portion of any one of claims 1 to 17.
22. A replicable expression vector capable, in a host cell, of expressing the DNA of claim 21.
23. A host cell transformed with a replicable expression vector of claim 22.
24. A derivative according to any one of claims 1 to 17 which comprises a peptide membrane binding element with low membrane affinity comprising one or more derivatisations selected from: a terminal cysteine residue optionally activated at the thiol group; an N-haloacetyl group (where halo signifies chlorine, bromine or iodine) located at the N-terminus of the peptide or at an ϵ -amino group of a lysine residue; an amide group at the C-terminus; an N-terminal blocking group; and a fatty acid N-acyl group at the N-terminus or at an ϵ -amino group of a lysine residue.

sequence of a peptide defined in claim 11 or 12 and a fatty acid N-acyl group of 8 to 18 methylene units at the N-terminus or at an ϵ -amino group of a lysine residue of the peptide.

26. A derivative according to claim 25 which comprises a C₁₀₋₂₀ fatty acyl derivative of an amino C₂₋₆ alkane thiol (optionally C-substituted).
27. A derivative according to claim 26 selected from N-(2-myristoyl) aminoethanethiol and N-myristoyl L-cysteine.
28. A pharmaceutical composition comprising a derivative according to any of claims 1 to 17 in combination with a pharmaceutically acceptable carrier or excipient.
29. A derivative according to any of claims 1 to 17 for use as a medicament in the treatment of a primary or secondary tumour.
30. A method of treatment of a primary or secondary tumour which comprises administering a soluble derivative of said soluble peptide according to any of claims 1 to 17.
31. The use of a derivative according to any of claims 1 to 17 for the preparation of a medicament for treatment of a primary or secondary tumour.
32. A derivative according to any one of claims 1 to 17 for use in the manufacture of a medicament for the treatment of a primary or secondary tumour.
33. A process for producing a soluble derivative according to any one of claims 1 to 17 which comprises covalently attaching a soluble anti-angiogenic peptide to a plurality of membrane binding elements.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/11, 9/68, 9/72, C07K 14/775, C12N 9/74, C07K 14/78, 14/575, A61K 47/48, C07K 16/36		A3	(11) International Publication Number: WO 00/04052
			(43) International Publication Date: 27 January 2000 (27.01.00)
(21) International Application Number: PCT/GB99/02292 (22) International Filing Date: 16 July 1999 (16.07.99) (30) Priority Data: 9815505.4 16 July 1998 (16.07.98) GB (71) Applicant (for all designated States except US): ADPROTECH PLC [GB/GB]; Second Floor, Units 7 & 8, The Maltings, Green Drift, Royston, Herts. SG8 5DY (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SMITH, Richard, Anthony, Godwin [GB/GB]; Norfolk House, Haverhill Road, Horseheath, Cambridge CB1 6QR (GB). BRIGHT, Jeremy, Richard [GB/GB]; The Haven, Chesham Road, Berkamstead, Herts. HP4 3AA (GB). STEWARD, Michael [GB/GB]; Whitebeam Cottage, Thomas Walk, Littlebury Green, Saffron Walden, Essex CB11 4XE (GB). COX, Vivienne, Frances [GB/GB]; Bec Cottage, Pounsley Road, Dunton Green, Sevenoaks, Kent TN13 2XP (GB). (74) Agent: DAVIES, Jonathan, Mark; Reddie & Grose, 16 Theobalds Road, London WC1X 8PL (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 20 April 2000 (20.04.00)	
(54) Title: SOLUBLE DERIVATIVES OF ANTI-ANGIOGENIC PEPTIDES			
(57) Abstract			
<p>It has been found that derivatives of angiogenesis inhibiting proteins may be prepared in which a negative feedback process can be enhanced for therapeutic purposes and which can be targeted to cell membranes and sites of active angiogenesis particularly those of the vascular endothelium. The invention provides a soluble derivative of a polypeptide capable of inhibiting angiogenesis, said derivative comprising a combination of heterologous membrane binding elements covalently associated with the polypeptide so that the derivative acquires affinity for the surface of the vascular endothelium particularly that of growing blood vessels. The soluble polypeptide may be selected from the non-catalytic regions of human plasminogen (within the N-terminal 560 residues of that protein); fragments thereof, particularly those generated by metalloprotease digestion of plasminogen; fragments of related proteins containing kringle domains such as hepatocyte growth factor or apolipoprotein (a), prothrombin, tissue-type plasminogen activator, urinary-type plasminogen activator and hybrids thereof with plasminogen sequences; mutants of the above kringle domains, those containing positively charged to neutral or negatively charged mutations at positions 20, 21, 78 and 79; fragments of collagen, particularly collagen XVIII; fragments of prolactin, the 16kDa N-terminal region of prolactin; neutralising antibodies against receptors for angiogenic mediators; antagonists of integrins involved in angiogenesis; and hybrids, derivatives or muteins thereof. Each membrane binding element with low membrane affinity may have a dissociation constant of 1μM–1mM, and the derivative may incorporate sufficient elements with low affinities for membrane components to result in a 0.01 – 10nM dissociation constant affinity for specific membranes.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

International Application No.

PC1/GB 99/02292

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/11 C12N9/68 C12N9/72 C07K14/775 C12N9/74
C07K14/78 C07K14/575 A61K47/48 C07K16/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 02454 A (ADPROTECH) 22 January 1998 (1998-01-22) cited in the application the whole document -----	1-33



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

10 January 2000

Date of mailing of the international search report

19/01/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040. Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Masturzo, P

Information on patent family members

PC1/GB 99/02292

Form PCT/ISA/210 (patent family annex) (July 1992)